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Review Article—

Evolution of Highly Pathogenic Avian Influenza H5N1 Virus in Natural Ecosystems of Northern Eurasia (2005–08)

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SUMMARY. Fifty-four strains of H5N1 highly pathogenic avian influenza (HPAI) virus were isolated from wild birds in the ecosystems of northern Eurasia and from poultry in the south of western Siberia (July 2005), at the mouth of Volga River (November 2005), at Uvs-Nur Lake on the boundary of the Great Lakes Depression in western Mongolia and the Tyva Republic of Russia (June 2006), in the vicinity of Moscow (February 2007), in the southeastern part of the Russian Plain (September 2007 and December 2007), and in the far east (April 2008) of the Russian Federation and were phenotypically characterized and deposited into the Russian state collection of viruses. Complete genome nucleotide sequences for 24 strains were obtained and deposited into GenBank. In all cases when strains were isolated from both wild birds and poultry in the same outbreak these strains were genetically closely related to each other. Until 2008 all HPAI H5N1 strains isolated in northern Eurasia clustered genetically with the viruses from Kukunor Lake (Qinghai Province, China), known as genotype 2.2 or the "Qinghai-Siberian" genotype. The viruses from the Qinghai-Siberian genotype have continued to evolve from those initially introduced into western Siberia in 2005 into two genetic groups: "Iran–North Caucasian" and "Tyva-Siberian." *In vitro* replication potential (50% tissue-culture infectious dose in porcine embryo kidney) of Qinghai-Siberian strains decreased over time, which could reflect decreasing virulence. Comparison of genome sequences with biological characteristics of the respective strains permitted us to identify point mutations in PB2, PB1, PA, HA, NP, NA, M2, NS1, and NS2 that possibly influenced the level of replication potential. The HPAI H5N1 virus, which penetrated into the south of the Russian Far East in spring 2008, belonged to genotype 2.3.2.

RESUMEN. *Estudio Recapitulativo*—Evolución de los virus de la influenza aviar de alta patogenicidad H5N1 en ecosistemas naturales del Norte de Eurasia (2005-08).

Cincuenta y cuatro cepas del virus de la influenza aviar de alta patogenicidad subtipo H5N1 fueron aisladas de aves silvestres en los ecosistemas del norte de Eurasia y de aves de corral del sur de Siberia occidental (julio de 2005), de la desembocadura del Río Volga (Noviembre de 2005), en el Lago Uvs al-Nur ubicado en el límite de la Depresión de los Grandes Lagos del Oeste de Mongolia y de la República de Tuvá en Rusia (Junio de 2006), de las cercanías de Moscú (Febrero del 2007), en la parte sureste de la Llanura Rusa (septiembre de 2007 y diciembre de 2007), y en el Extremo Oriente de Rusia (Abril de 2008). Las cepas fueron caracterizadas fenotípicamente y se depositaron en la colección estatal rusa de virus. Las secuencias completas de nucleótidos del genoma de 24 cepas fueron obtenidas y depositadas en el GenBank. En todos los casos, cuando las cepas fueron aisladas de aves silvestres y de aves de corral del mismo brote, estas cepas resultaron estar estrechamente relacionadas entre sí genéticamente. Hasta el año 2008, todas las cepas de influenza aviar altamente patógenas H5N1 aisladas en el norte de Eurasia se agruparon genéticamente con el virus del Lago Kukunor (provincia de Qinghai, China), conocido como genotipo 2.2 o el genotipo "Qinghai-siberiano". Los virus del genotipo Qinghai-siberiano han continuado su evolución a partir de los virus que inicialmente se introdujeron en Siberia en el 2005 resultando en dos grupos genéticos: el "Irán-Cáucaso del Norte" y el "Tuvá-Siberiano". El potencial de replicación *in vitro* de las cepas Qinghai-Siberianas (en dosis infectantes 50% en cultivo de tejidos de células de porcino embrionarias) disminuyó con el tiempo, lo que podría reflejar una disminución en la virulencia. La comparación de las secuencias del genoma con las características biológicas de las cepas respectivas ha permitido identificar mutaciones puntuales en PB2, PB1, PA, HA, NP, NA, M2, NS1 y NS2 que posiblemente influyeron en el nivel del potencial de replicación. El virus de la influenza aviar altamente patógena H5N1, que penetró en el sur del Extremo Oriente de Rusia en la primavera de 2008, pertenecían al genotipo 2.3.2.

Key words: virus, HPAI, H5N1, natural ecosystems, northern Eurasia, evolution

Abbreviations: AIV = avian influenza virus; BHK-21 = baby hamster kidney cell line; HA = hemagglutinin; HPAI = highly pathogenic avian influenza; LECH = human embryo lung cell line; LPAI = low pathogenicity avian influenza; MDCK = Madin-Darby canine kidney cell line; NA = neuraminidase; NLS = nuclear location signal; RNP = ribonucleoproteide; RSCV = Russian state collection of viruses; RT-PCR = reverse transcription followed by polymerase chain reaction; SPEV = porcine embryo kidney cell line; $TCID_{50}$ = 50% tissue-culture infectious dose; Vero E6 = clone E6 of vervet-obtained monkey kidney cell line

The richest gene pool of influenza A virus is among wild waterfowl, and the ability of influenza viruses to reassort provides a vast array of possible variants. Virus variants circulating in bird populations could be progenitors for the emerging and reemerging epizootic and pandemic viruses. It is why 45 yr ago we started doing surveillance of avian influenza virus (AIV) circulation in key points on the main migration routes of birds in northern Eurasia. During this time more than a thousand influenza A virus strains belonging to 15 of the 16 known HA subtypes were isolated in different ecosystems of northern Eurasia (17,18,20,21,30,31,35,38,39) (Fig. 1). The HA subtypes of the strains isolated from teal in 2001 in the Russian Far East and

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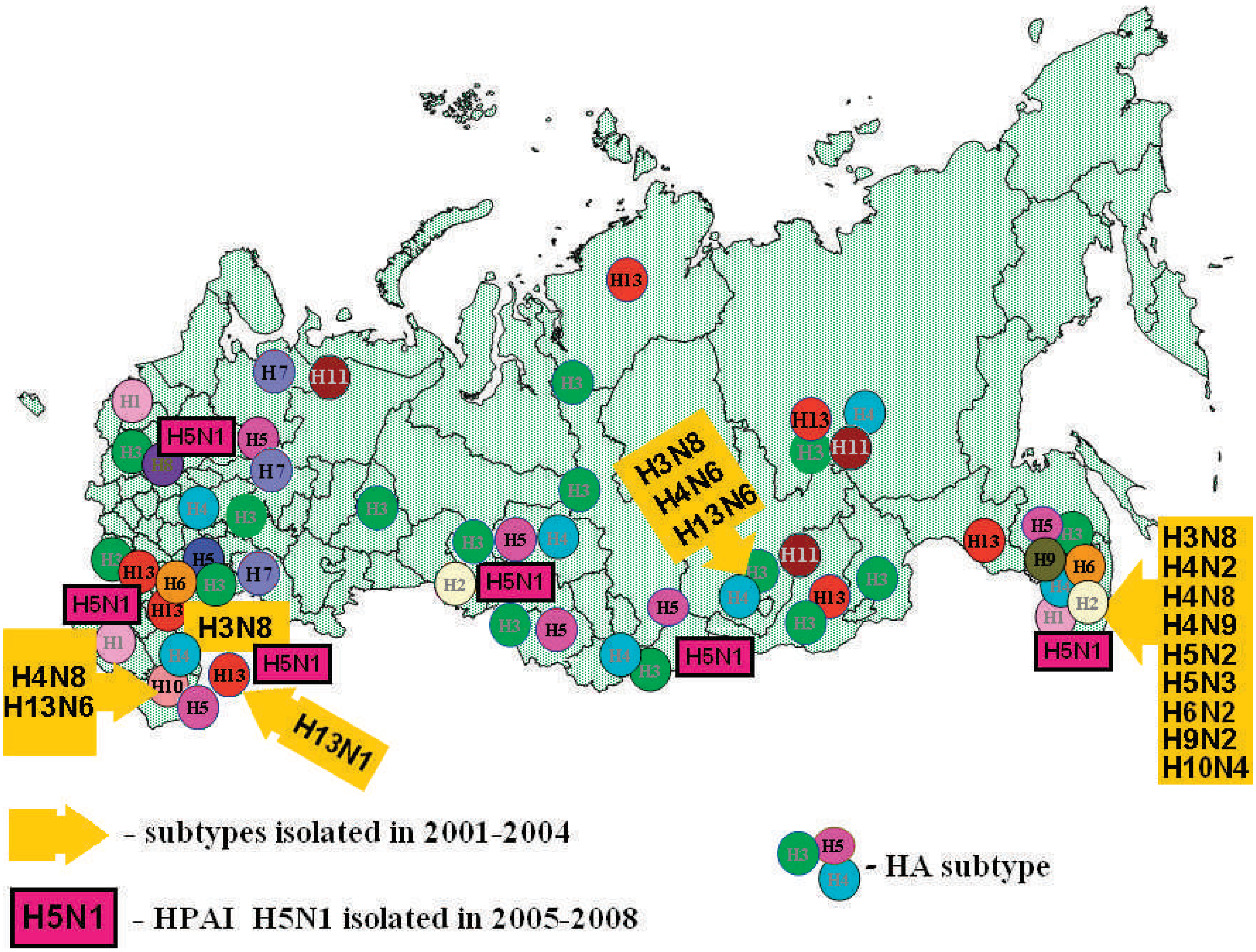

Fig. 1. Influenza A virus subtypes isolated from birds in natural and anthropogenic ecosystems of northern Eurasia (1962–2008).

eastern Siberia were shown to be genetically related to the H5 strains isolated from poultry in 1997 in Italy. The AIV strains from mallard isolated in 1991 in the Altai region of southwestern Siberia were similar to A/duck/Malaysia/F119-3/97 with a HA cleavage site that is characteristic of low pathogenicity avian influenza (LPAI) strains (18,30,31,33).

Development and integration of modern virologic, ecologic, and genetic techniques could allow early detection of viruses that are a threat to both veterinary and public health and promote efforts to mitigate new outbreaks. In the former USSR, there was a system for wide-scale monitoring of natural foci of viral infections that was coordinated by the Virus Ecology Center of the D. I. Ivanovsky Institute of Virology (Russian Academy of Medical Sciences, Moscow, Russia). This monitoring network has allowed us to document the introduction of the current H5N1 epizooty into northern Eurasia, and with modern methods we have biologically and molecularly characterized these newly introduced viruses. In the present article we discuss results obtained at the Influenza Ecology and Epidemiology Center as a result of studying HPAI H5N1 from the time of its introduction into the ecosystems of northern Eurasia in spring of 2005 until now.

MATERIALS AND METHODS

Collection of field materials (cloacal/tracheal swabs, brain, liver, lungs) from sick and dead birds was performed during epizootic outbreaks in northern Eurasia during 2005–08. Each sample was placed into a 2-ml tube with 40% glycerol in a phosphate buffer solution (pH 7.4), stored, and transported to the laboratory in liquid nitrogen in Dewar vessels.

Reverse transcription followed by polymerase chain reaction (RT-PCR) for the detection of RNA of NP, M, and HA genes from H5 of the influenza A virus was conducted according to the standard techniques using specific primers (NARVAC, Russia) (11).

Isolation and investigation of replication potential *in vitro* of virus strains was performed using either 9-to-10-day-old specific-pathogen-free chicken eggs or continuous cell culture lines: porcine embryo kidney (SPEV), Madin-Darby canine kidney (MDCK), baby hamster kidney (BHK-21), human embryo lung (LECH), or clone E6 of vervet-obtained monkey kidney (Vero E6) (6).

Decrease of the *in vitro* replication potential (50% tissue-culture infectious dose [$TCID_{50}$] in SPEV) over time was characterized by angulation of the best fit line calculated by the least squares method.

Identification of isolated virus strains was carried out by RT-PCR, hemagglutination inhibition testing, neutralization tests according to the standard techniques, and biologic microchips (Biochip-IMB Ltd., V.A. Engelhardt Institute of Molecular Biology, Moscow, Russia) according to the manufacturer's recommendations.

Nucleotide sequencing was performed using the automated sequencing apparatus ABI prism 3130 (Applied Biosystems, Foster City, CA) according to the manufacturer's recommendations.

Comparative analysis of nucleotide sequences was carried out using the commercial software package DNASTAR (DNASTAR Inc., Madison, WI), as well as our own information techniques based on

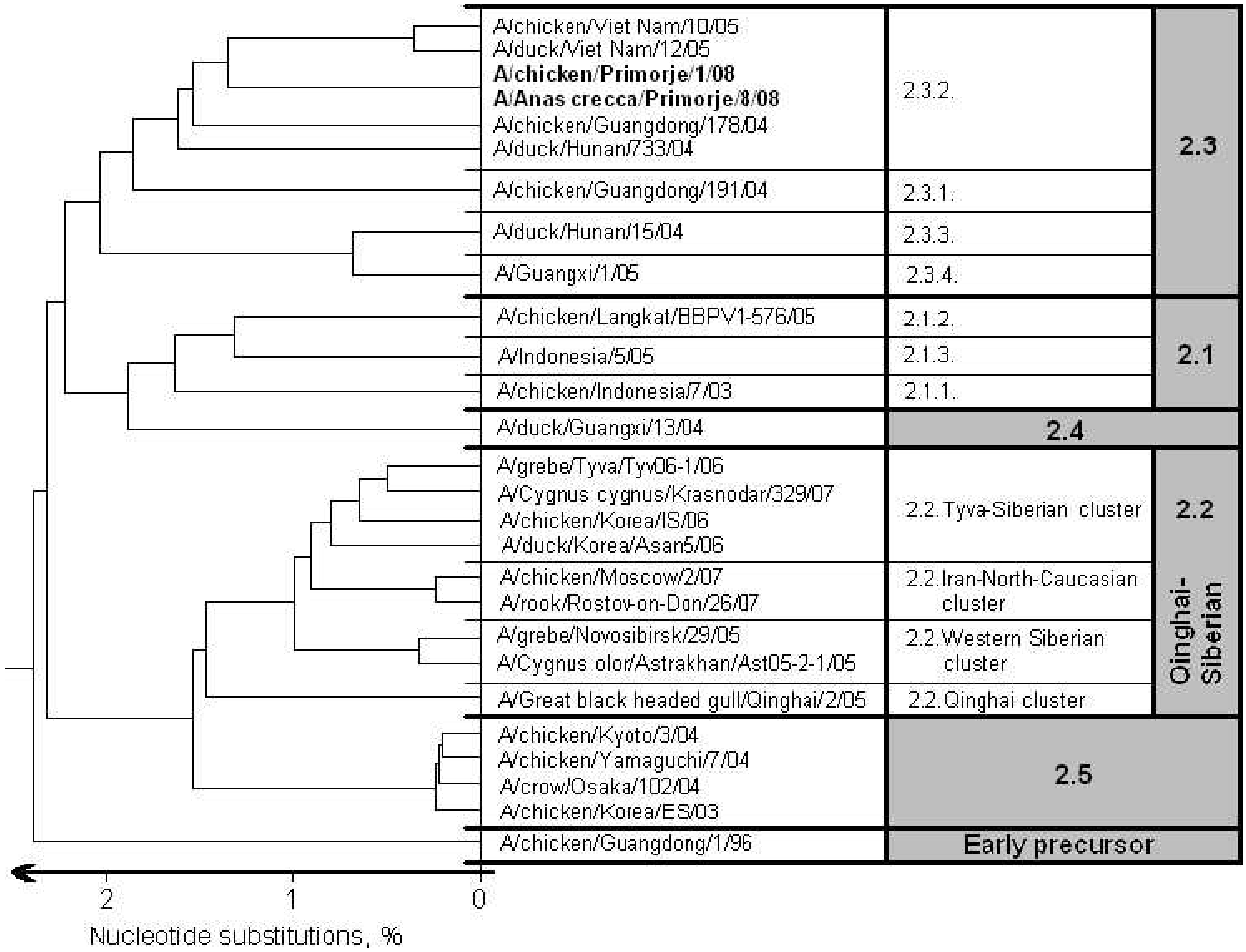

Fig. 2. Phylogenetic analysis of complete nucleotide sequences of open reading frame for HPAI H5N1 HA.

Statistics 6.0 (StatSoft Inc., Tulsa, OK), ArcView 3.2 (ESRI Inc., Redlands, CA), and MatLab 6.0 (Softline Inc., Berkeley, CA).

RESULTS

After the introduction of the HPAI H5N1 virus into northern Eurasia in the spring of 2005 until 2008, we have isolated 54 strains (27 from wild birds, 27 from poultry) of this virus from epizootic outbreaks in the following locations: in the south of western Siberia (July 2005) (22,25,36); at the mouth of the Volga River (November 2005) (23); at Uvs-Nur Lake on the boundary of the Great Lakes Depression in western Mongolia and the Tyva Republic of Russia (June 2006) (24); in the vicinity of Moscow (February 2007) (27); in the northeastern part of the Azov Sea basin (September 2007) (26); in the southwestern part of the Russian Plain (December 2007) (28); and in the Russian Far East (April 2008) (29). All strains were phenotypically characterized and deposited into Russian state collection of viruses (RSCV) (Table 1). The nucleotide sequences of all eight gene segments for 24 strains were deposited into GenBank (Table 2).

All isolated strains appeared to belong to the Asian H5N1 genotypic clade 2.2 with the exception of four strains belonging to genotype clade 2.3.2, which were isolated in the Far East in the spring of 2008 (Table 1; Fig. 2). The clade 2.2 avian influenza viruses that were introduced into northern Eurasia (22,25,36) were most closely related to viruses responsible for the mass mortality in wild birds—mainly among bar-headed geese (*Eulabeia indica*) and great black-headed gulls (*Larus ichthyaetus*)—at Kukunor Lake in April of 2005 (Qinghai Province, China) (5,14). The virus lineage appeared to move north with the wild bird migration defining the genotype 2.2 viruses as the "Qinghai-Siberian" clade.

The main genetic characteristics of Qinghai-Siberian clade (22) persisted as the virus lineage extended into the western part of northern Eurasia (1,2,12,22,23,24,25,26,27,28) and Africa (2005–08) (2,3,10). The other genes of the Qinghai-Siberian genotype are associated with group Z, which has dominated among poultry in southeastern Asia since 2003–04 (4). The group Z genotype has several unique genetic markers including a 20-mer amino acid fragment deletion C_{49}NQSIITYENNTWVNQTYVN$_{68}$ in the N1 gene compared to A/goose/Guangdong/1996. The HA cleavage site—P_{337}QGERRRKKRGLF$_{349}$—has multiple basic amino acid insertions. Three types of silent nucleotide substitutions are known in the coding region of the cleavage site among the Qinghai-Siberian clade: $G \overset{1020}{\rightarrow} A$ (A/chicken/Volgograd/236/2006), $G \overset{1028}{\rightarrow} A$ (A/pied magpie/Liaoning/7/2005), and $A \overset{1044}{\rightarrow} G$ (A/chicken/Crimea/04/2005), as well as four types of amino acid substitutions: $G \overset{339}{\rightarrow} R$ (A/bar-headed goose/Qinghai/{1,3}/2005, A/brown-headed gull/Qinghai/1/2005, A/great black-headed gull/Qinghai/3/2005, A/great cormorant/Qinghai/3/2005, A/whooper swan/Mongolia/

Table 1. Infection activity *in vitro* of HPAI H5N1 strains isolated in natural and anthropogenic ecosystems of northern Eurasia (2005–08).

Date	Region	Ecologic group of birds	Strain[A]	RSCV deposition number	Clinical features[B]	$\log_{10}$ $TCID_{50}$/ml for SPEV
July 2005	South of western Siberia (Novosibirsk region) (19,24,35)	Wild	**A/grebe/Novosibirsk/29/2005**	2372	∅	5.7
			Mean value:			**5.7**
		Poultry	**A/duck/Novosibirsk/56/2005**	2371	⊗	7.7
			A/duck/Novosibirsk/67/2005	2376	⊕	10.2
			A/chicken/Novosibirsk/64/2005	2373	⊕	11.2
			A/chicken/Novosibirsk/65/2005	2374	⊕	10.7
			A/chicken/Novosibirsk/66/2005	2375	⊕	10.7
			Mean value:			**10.1**
November 2005	Mouth of Volga River (Astrakhan region, Kalmyk Republic) (22)	Wild	**A/Cygnus olor/Astrakhan/Ast05-2-1/2005**	2379	⊗	3.7
			A/Cygnus olor/Astrakhan/Ast05-2-2/2005	2380	⊗	4.2
			A/Cygnus olor/Astrakhan/Ast05-2-3/2005	2381	⊗	4.2
			A/Cygnus olor/Astrakhan/Ast05-2-4/2005	2382	⊗	3.7
			A/Cygnus olor/Astrakhan/Ast05-2-5/2005	2383	⊗	5.2
			A/Cygnus olor/Astrakhan/Ast05-2-6/2005	2384	⊗	5.2
			A/Cygnus olor/Astrakhan/Ast05-2-7/2005	2385	⊗	5.7
			A/Cygnus olor/Astrakhan/Ast05-2-8/2005	2386	⊗	4.2
			A/Cygnus olor/Astrakhan/Ast05-2-9/2005	2387	⊗	3.2
			A/Cygnus olor/Astrakhan/Ast05-2-10/2005	2388	⊗	4.7
			Mean value:			**4.4**
June 2006	Uvs-Nur Lake (Tyva Republic) (23)	Wild	**A/grebe/Tyva/Tyv06-1/2006**	2393	⊗	8.0
			A/grebe/Tyva/Tyv06-2/2006	2394	⊗	8.5
			A/cormorant/Tyva/Tyv06-4/2006	2396	∅	5.0
			A/coot/Tyva/Tyv06-6/2006	2397	⊗	5.0
			A/grebe/Tyva/Tyv06-8/2006	2395	⊕	8.0
			A/tern/Tyva/Tyv06-18/2006	2399	∅	5.0
			Mean value:			**6.6**
February 2007	Vicinity of Moscow (Moscow and Kaluga regions) (26)	Poultry	A/chicken/Moscow/1/2007	2403	⊕	4.0
			A/chicken/Moscow/2/2007	2404	⊕	4.5
			A/chicken/Moscow/3/2007	2405	⊕	4.0
			A/chicken/Moscow/4/2007	2406	⊕	4.0
			A/goose/Moscow/5/2007	2407	⊕	4.0
			A/chicken/Moscow/6/2007	2408	⊕	4.5
			A/chicken/Moscow/7/2007	2409	⊕	4.5
			A/chicken/Moscow/8/2007	2410	⊕	4.0
			A/chicken/Moscow/9/2007	2414	⊕	4.0
			Mean value:			**4.2**
September 2007	Northeastern part of Azov Sea basin (Krasnodar krai) (25)	Wild	**A/Cygnus cygnus/Krasnodar/329/2007**	2421	⊗	3.5
			Mean value:			**3.5**
		Poultry	**A/chicken/Krasnodar/300/2007**	2418	⊗	3.5
			A/chicken/Krasnodar/301/2007	2419	⊗	3.0
			A/chicken/Krasnodar/302/2007	2420	⊗	3.5
			Mean value:			**3.3**
December 2007	Southwestern part of Russian Plain (Rostov region) (27)	Wild	**A/pigeon/Rostov-on-Don/6/2007**	2423	∅	6.5
			A/pigeon/Rostov-on-Don/7/2007	2424	∅	5.5
			A/heron/Rostov-on-Don/11/2007	2425	∅	6.0
			A/pigeon/Rostov-on-Don/21/2007	2426	∅	6.0
			A/rook/Rostov-on-Don/26/2007	2427	∅	6.5
			A/rook/Rostov-on-Don/27/2007	2428	∅	6.0
			A/tree sparrow/Rostov-on-Don/28/2007	2429	∅	6.0
			A/starling/Rostov-on-Don/39/2007	2435	∅	6.0
			Mean value:			**6.1**
		Poultry	A/chicken/Rostov-on-Don/31/2007	2430	⊗	7.5
			A/chicken/Rostov-on-Don/32/2007	2431	⊗	7.0

Table 1. Continued.

Date	Region	Ecologic group of birds	Strain[A]	RSCV deposition number	Clinical features[B]	$\log_{10}$ $TCID_{50}$/ml for SPEV
			A/chicken/Rostov-on-Don/33/2007	2432	$\oplus$	7.0
			A/chicken/Rostov-on-Don/34/2007	2433	$\oplus$	7.5
			A/chicken/Rostov-on-Don/35/2007	2434	$\oplus$	7.0
			A/muscovy duck/Rostov-on-Don/51/2007	2436	$\oplus$	7.0
			A/chicken/Rostov-on-Don/52/2007	2437	$\oplus$	7.5
			Mean value:			**7.2**
April 2008	Suifun-Khanka Lowland (Primorsky krai) (28)	Wild	A/Anas crecca/Primorje/8/2008	2441	$\emptyset$	4.0
			Mean value:			**4.0**
		Poultry	**A/chicken/Primorje/1/2008**	2440	$\oplus$	4.5
			A/chicken/Primorje/11/2008	2442	$\oplus$	4.0
			A/chicken/Primorje/12/2008	2443	$\oplus$	4.5
			Mean value:			**4.3**

[A]Strains with complete genome sequences (Table 2) are marked by bold font.
[B]$\emptyset$ = birds without clinical features; $\otimes$ = birds with clinical features; $\oplus$ = dead birds.

13/2005); $\mathrm{R}\overset{341}{\rightarrow}\mathrm{G}$ (A/chicken/Sudan/1784-{7,10}/2006); $\mathrm{R}\overset{343}{\rightarrow}\mathrm{K}$ (A/pied magpie/Liaoning/7/2005); $\mathrm{K}\overset{344}{\rightarrow}\mathrm{R}$ (A/whooper swan/Mongolia/7/2005). The presence of consensus G_{339} makes the Qinghai-Siberian clade different from other HPAI H5N1 variants from southeastern Asia containing consensus R_{339}. The highest portion of R_{339}—5/15 (33.3%)—was detected in the initial outbreak at Kukunor Lake (Qinghai, China). This suggests that the Qinghai-Siberian strains that originated from southeastern Asia were under a "bottleneck" selection at the early stage of their evolution. The NS1 protein has E_{92}, instead of the D_{92} that is common for virus variants from birds (13,15), and a five–amino acid deletion.

The Qinghai-Siberian clade includes viruses that have infected and caused severe disease and mortality in humans, but currently the virus does not appear to transmit efficiently in humans. We analyzed representative viruses in our collection for their potential to replicate in mammals. We found that isolated strains replicated effectively in mammalian cell culture lines BHK-21, LECH, Vero E6, MDCK, and SPEV (6,25). The PB2 has a consensus K_{627} that promotes virulence in mammalian cells (4,16). Six representative isolates from the Qinghai-Siberian clade have E_{627}, including A/bar-headed goose/Qinghai/2/2005, A/ruddy shelduck/Qinghai/1/2005, A/duck/Novosibirsk/02/2005, A/duck/Kurgan/08/2005, A/Cygnus olor/Astrakhan/Ast05-2-4/2005, and A/Cygnus olor/Italy/808/2006. These strains are uniformly distributed through time and territory as the result of stochastic nature of E_{627} and the absence of a tendency for K_{627} elimination. Substitutions that are correlated with virus tropism in mammals have been identified, including $\mathrm{D}\overset{701}{\rightarrow}\mathrm{N}$ in PB2 (4,10), $\mathrm{S}\overset{714}{\rightarrow}\mathrm{R}$ in PB2, $\mathrm{L}\overset{13}{\rightarrow}\mathrm{P}$ in PB1, $\mathrm{S}\overset{678}{\rightarrow}\mathrm{N}$ in PB1, and $\mathrm{K}\overset{615}{\rightarrow}\mathrm{N}$ in PA (8). In the viruses of the Qinghai-Siberian clade, proline is present in all the genomes in the 13th aa position of PB1, and asparagine at position 701 of the PB2 protein was found only in A/ruddy shelduck/Qinghai/1/2005.

Based on the amino acid sequence of HA receptor-binding sites of Qinghai-Siberian isolates we predict its affinity for $\alpha 2'$-$3'$-sialic acids common for intestinal avian cells (*vs.* $\alpha 2'$-$6'$-sialic acids of cells in the human respiratory tract) (9,34) containing E_{202}, Q_{238}, and G_{240} using aa numbering from the beginning of the protein including the leader sequence. Double mutation, $\mathrm{Q}\overset{238}{\rightarrow}\mathrm{L}$ and $\mathrm{G}\overset{240}{\rightarrow}\mathrm{S}$, or just the single mutation of $\mathrm{E}\overset{202}{\rightarrow}\mathrm{D}$ could switch HA receptor-binding affinity from avian to human receptors (34).

All the Qinghai-Siberian isolates are sensitive to amantadine, rimantadine, and oseltamivir, which has been confirmed by both direct biological experiments *in vitro* (19) and the presence (22,23,24,25,26,27,28) of marker substitutions (16,32,40) in M and NA virus proteins.

Decrease of *in vitro* reproduction potential of isolated strains (Fig. 3) is more evident for poultry ($TCID_{50} = 11.847 - 0.272 \times t$) in respect to wild birds ($TCID_{50} = 6.185 - 0.066 \times t$), where t is time expressed in months starting from the beginning of 2005. Table 3 facilitates comparison of phenotypic changes with point mutations in the proteins of HPAI H5N1/2.2 strains isolated in northern Eurasia.

Strains isolated from wild birds and poultry (Table 1) in Primorje (Far East) in April 2008 were found to be different from the clade 2.2 (Qinghai-Siberian) genotype and belonged to the clade 2.3.2. The A/chicken/Primorje/1/2008 and A/Anas crecca/Primorje/8/2008 were identical. The closest neighbor of Primorje 2008 strains are A/chicken/Viet Nam/10/2005 (nucleotide sequence similarity for HA gene is 97.5%), A/chicken/Guandong/178/2004 (97.3%), and A/duck/Viet Nam/12/2005 (97.2%). The cleavage site of HA—$P_{337}QRERRRKRGLF_{348}$—contained multiple basic amino acid motifs, which is typical for HPAI, but differs from the Qinghai-Siberian HA cleavage site. The 2008 isolates also belonged to group Z for the internal genes, had avian-type receptor specificity, were sensitive for M2-channel formation, and were neuraminidase inhibitors. Nevertheless, in contrast with HPAI H5N1/2.2 strains, which contained K_{627} (which promotes virulence in mammalian cells), the Far Eastern 2008 isolates contained E_{627} typical for avian-adaptive variants. Reduced tropism for mammalian cell lines was also verified by direct experiments *in vitro* (29).

A comparison of the biologic biochip of a representative isolate from the clade 2.2 and from 2.3.2 isolates show a clear difference in hybridization pattern in the hemagglutinin and neuraminidase genes (Fig. 4). The sequence similarity between A/chicken/Primorje/1/2008 and A/Anas crecca/Primorje/8/2008 *vs.* HA H5/2.2 strains are 92.9%–95.3% for HA and 94.1%–95.3% for NA nucleotide sequences. These differences are reflected by nucleotide probes of the biologic microchip that leads to different hybridization pattern for HA H5/2.2 and HA H5/2.3.2 in Fig. 4.

DISCUSSION

Northern Eurasia has the world's largest nesting area for migratory birds and it connects with Southeast Asia, Middle Asia, India, the Middle East, Africa, North America, and the Pacific islands by migration pathways (20). Every year large populations of nonim-

Table 2. Identification numbers of GenBank for HPAI H5N1 strains isolated in natural and anthropogenic ecosystems of northern Eurasia (2005–08) with complete nucleotide sequences of genomes (marked by bold font in Table 1).

Strain	HA genotype	Source of isolation	Complete nucleotide sequences							
			PB2	PB1	PA	HA	NP	NA	M	NS
A/grebe/Novosibirsk/29/05	2.2	Great crested grebe (*Podiceps cristatus*)	DQ232607	DQ232605	DQ234075	DQ230521	DQ232609	DQ230523	DQ234077	DQ234073
A/duck/Novosibirsk/56/05	2.2	Domestic duck (*Anas platyrhynchos domesticus*)	DQ232608	DQ232606	DQ234076	DQ230522	DQ232610	DQ230524	DQ234078	DQ234074
A/Cygnus olor/Astrakhan/Ast05-2-1/05	2.2	Mute swan (*Cygnus olor*)	DQ389161	DQ394578	DQ394579	DQ389158	DQ394577	DQ389159	DQ394576	DQ389160
A/Cygnus olor/Astrakhan/Ast05-2-2/05	2.2	Mute swan (*Cygnus olor*)	DQ343506	DQ343505	DQ343504	DQ343502	DQ359694	DQ343503	DQ359692	DQ359693
A/Cygnus olor/Astrakhan/Ast05-2-3/05	2.2	Mute swan (*Cygnus olor*)	DQ358750	DQ358749	DQ358748	DQ358746	DQ358751	DQ358747	DQ358739	DQ358752
A/Cygnus olor/Astrakhan/Ast05-2-4/05	2.2	Mute swan (*Cygnus olor*)	DQ363916	DQ363915	DQ363917	DQ363918	DQ363929	DQ363919	DQ363925	DQ363926
A/Cygnus olor/Astrakhan/Ast05-2-5/05	2.2	Mute swan (*Cygnus olor*)	DQ365011	DQ365008	DQ365007	DQ365004	DQ365006	DQ365005	DQ365009	DQ365010
A/Cygnus olor/Astrakhan/Ast05-2-6/05	2.2	Mute swan (*Cygnus olor*)	DQ365001	DQ365000	DQ364999	DQ364996	DQ364998	DQ364997	DQ365002	DQ365003
A/Cygnus olor/Astrakhan/Ast05-2-7/05	2.2	Mute swan (*Cygnus olor*)	DQ363921	DQ363920	DQ363922	DQ363923	DQ363930	DQ363924	DQ363928	DQ363927
A/Cygnus olor/Astrakhan/Ast05-2-8/05	2.2	Mute swan (*Cygnus olor*)	DQ386305	DQ386304	DQ399537	DQ399540	DQ399539	DQ399541	DQ399542	DQ399538
A/Cygnus olor/Astrakhan/Ast05-2-9/05	2.2	Mute swan (*Cygnus olor*)	DQ399543	DQ406738	DQ406737	DQ399547	DQ399545	DQ399546	DQ400912	DQ399544
A/Cygnus olor/Astrakhan/Ast05-2-10/05	2.2	Mute swan (*Cygnus olor*)	DQ434890	DQ423612	DQ434891	DQ434889	DQ440580	DQ440579	DQ434888	DQ434887
A/grebe/Tyva/Tyv06-1/06	2.2	Great crested grebe (*Podiceps cristatus*)	DQ914807	DQ914810	DQ978999	DQ914808	DQ916293	DQ914809	DQ914805	DQ914806
A/grebe/Tyva/Tyv06-2/06	2.2	Great crested grebe (*Podiceps cristatus*)	DQ852607	DQ852606	DQ852603	DQ852600	DQ852602	DQ852601	DQ852604	DQ852605
A/grebe/Tyva/Tyv06-8/06	2.2	Great crested grebe (*Podiceps cristatus*)	DQ863510	DQ863509	DQ863508	DQ863503	DQ863506	DQ863507	DQ863504	DQ863505
A/chicken/Moscow/2/07	2.2	Chicken (*Gallus gallus domesticus*)	EF474443	EF474444	EF474445	EF474450	EF474447	EF474448	EF474449	EF474446
A/chicken/Krasnodar/300/07	2.2	Chicken (*Gallus gallus domesticus*)	EU163436	EU163435	EU163434	EU163431	EU163432	EU163433	EU163429	EU163430
A/Cygnus cygnus/Krasnodar/329/07	2.2	Whooper swan (*Cygnus cygnus*)	EU257707	EU257636	EU257637	EU257631	EU257635	EU257632	EU257633	EU257634
A/pigeon/Rostov-on-Don/6/07	2.2	Rock dove (*Columba livia*)	EU441930	EU441931	EU441932	EU441937	EU441933	EU441936	EU441935	EU441934
A/rook/Rostov-on-Don/26/07	2.2	Rook (*Corvus frugilegus*)	EU814510	EU814509	EU814508	EU814503	EU814506	EU814505	EU814504	EU814507
A/starling/Rostov-on-Don/39/07	2.2	Starling (*Sturnus vulgaris*)	EU486848	EU486849	EU486850	EU486855	EU486851	EU486854	EU486853	EU486852
A/chicken/Rostov-on-Don/35/07	2.2	Chicken (*Gallus gallus domesticus*)	EU414265	EU420032	EU408333	EU401751	EU401754	EU401753	EU401752	EU401755
A/muscovy duck/Rostov-on-Don/51/07	2.2	Muscovy duck (*Cairina moschata*)	EU441922	EU441923	EU441924	EU441929	EU441925	EU441928	EU441927	EU441926
A/chicken/Primorje/1/08	2.3.2	Chicken (*Gallus gallus domesticus*)	EU672455	EU672456	EU672457	EU676174	EU672458	EU672460	EU676173	EU672459

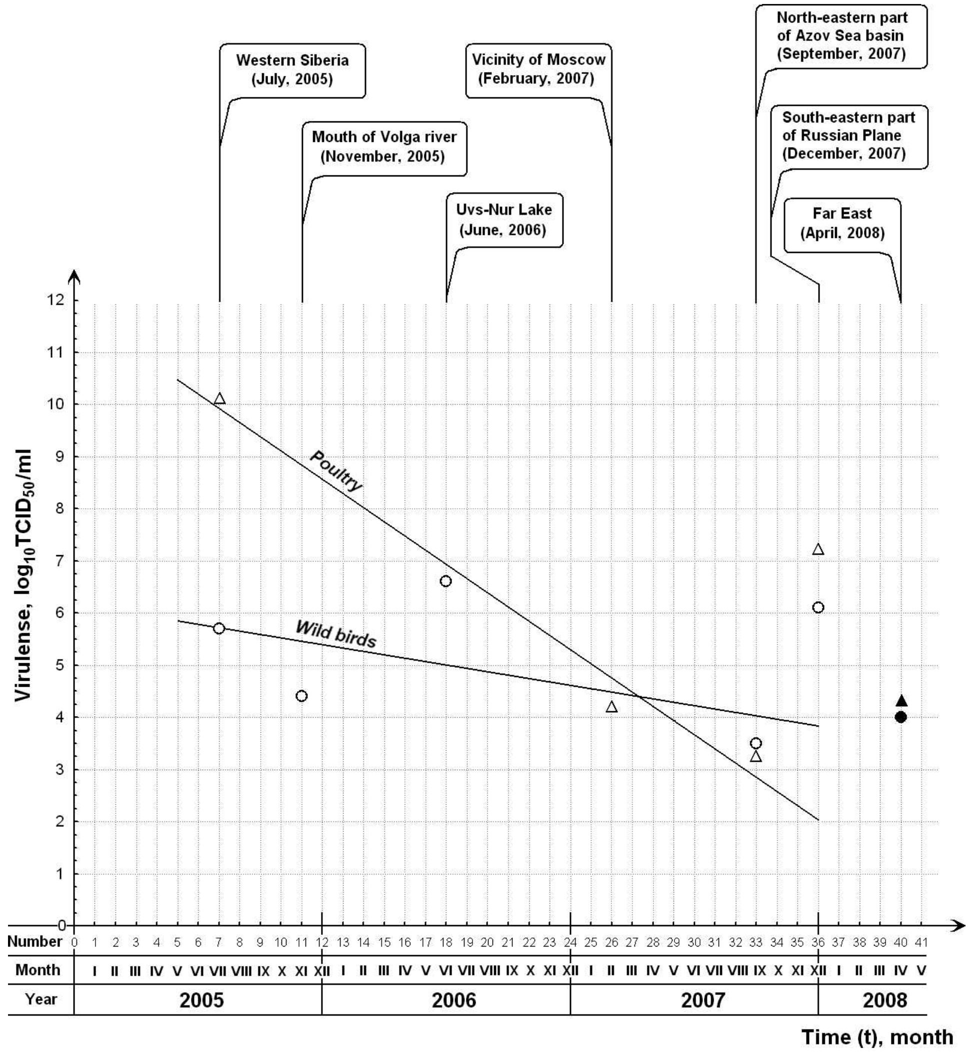

Fig. 3. Virulence dynamics of HPAI H5N1 strains isolated in northern Eurasia (2005–08): ○ = wild birds; △ = poultry; ● = genotype HA/H5/2.2; ▲ = genotype HA/H5/2.3.2. Epizooty in December 2007 was excluded from the trend calculation because of distinctive ecologic features described in the text.

mune juvenile birds are infected with one or more different influenza viruses with the potential generation of new virus variants, which are under intensive selective pressure in overwintering areas. On the eve of HPAI H5N1 epizooty starting in the autumn of 2003 we warned about the likelihood for outbreaks with the Asian lineage HPAI H5N1 at the International Conference "Options for the Control of Influenza V" (Okinawa, Japan, October 4–13, 2003) (31).

A second prediction was that overwintering migrating birds could transmit the HPAI virus into northern Eurasia during the spring migration. We discussed two possible scenarios of this introduction: through Dzhungarian (crossing with the Indian-Asian migration route) and via the Asian-Pacific migration routes. Preparing for these possible events we started doing increased surveillance in the south of western Siberia (Russian Foundation for Basic Research Project 03-a04-49158) and in Primorje (International Science-Technical Center Project 2800) in the spring of 2004. In April of 2005 a wide epizootic outbreak emerged at Kukunor Lake (Qinghai Province, China) and from this location we predicted the virus could spread at the Dzhungarian Gate, which links northwestern spurs of Tibet and West Siberian Lowland. The HPAI H5N1 epizooty, which emerged initially in the Chany Lake Depression in July 2005 and then spread to the south of western Siberia in August 2005, verified our prediction. This epizooty was likely introduced through the movements of infected wild birds and spread to poultry as a result of direct or indirect (water contamination) interactions. This was facilitated by the location of villages and poultry production facilities surrounded by wide marshes. Sick poultry (see Fig. 5A) had extremely high temperatures, depression, diarrhea, plumage shedding, corneal opacity, conjunctivitis, cerebral hemorrhages, lesions on viscera, and hemorrhages in mucosa, viscera and muscles (25,36). The viruses isolated in Siberia from wild birds and poultry (July 2005; Tables 1, 2) together with Qinghai strains (May 2005) (5,14) formed a separate clade in the Asian H5N1 lineage and demonstrated how quickly this lineage of virus could move within the region. The last process was facilitated by the presence of large populations of naive waterfowl that were going to migrate throughout overwintering areas.

The third prediction was that the virus would move with the migrating birds to the overwintering locations. In accordance with this prediction epizootic outbreaks occurred along the main migration routes in the Urals, the Russian Plain, Europe, Africa, Middle Asia, and India (1,2,3,10,12,23,37). In November 2005, the epizooty with mass deaths emerged in the downstream part of the mouth of the Volga River among the local population of mute swans

Table 3. Point mutations in the proteins of HPAI H5N1/2.2 strains isolated in northern Eurasia (2005–08).

A

Strains*	Amino acid substitutions** NP	NA	M1	M2	NS1	NS2
A/{*}/05 ↓ A/{*}/{06–07}	Y_{10} ↓ $\mathbf{H_{10}}$; N_{397} ↓ $\mathbf{S_{397}}$	no	no	no	D_{202} ↓ $\mathbf{G_{202}}$	M_{50} ↓ $\mathbf{V_{50}}$; I_{60} ↓ $\mathbf{T_{60}}$
A/duck/Novosibirsk/56/05 ↓ A/grebe/Novosibirsk/29/05	$\boxed{\mathbf{T_{403}}}$ ↓ A_{403}	M_{29} ↓ $\boxed{\mathbf{I_{29}}}$; $\boxed{\mathbf{L_{143}}}$ ↓ V_{143}	no	no	no	no
A/duck/Novosibirsk/56/05 ↓ A/Cygnus olor/Astrakhan/Ast05-2-{1-10}/05	$\mathbf{T_{403}}$ ↓ A_{403}	$\mathbf{L_{143}}$ ↓ V_{143}	no	no	no	no
A/duck/Novosibirsk/56/05 ↓ A/grebe/Tyva/Tyv06-{1,2,8}/06	Y_{10} ▼ $\boxed{\mathbf{H_{10}}}$; N_{397} ▼ $\boxed{\mathbf{S_{397}}}$; $\mathbf{T_{403}}$ ↓ A_{403}	$\mathbf{L_{143}}$ ↓ V_{143}; P_{320} ▼ $\boxed{\mathbf{L_{320}}}$	no	no	D_{202} ▼ $\boxed{\mathbf{G_{202}}}$	M_{50} ▼ $\boxed{\mathbf{V_{50}}}$; I_{60} ▼ $\boxed{\mathbf{T_{60}}}$
A/duck/Novosibirsk/56/05 ↓ A/chicken/Moscow/2/07	Y_{10} ▼ $\mathbf{H_{10}}$; K_{90} ▼ $\boxed{\mathbf{R_{90}}}$; A_{373} ▼ $\boxed{\mathbf{T_{373}}}$; N_{397} ▼ $\mathbf{S_{397}}$; $\mathbf{T_{403}}$ ↓ A_{403}	R_{44} ↓ $\boxed{\mathbf{C_{44}}}$; $\mathbf{L_{143}}$ ↓ V_{143}	no	no	D_{202} ▼ $\mathbf{G_{202}}$	M_{50} ▼ $\mathbf{V_{50}}$; I_{60} ▼ $\mathbf{T_{60}}$
A/duck/Novosibirsk/56/05 ↓ A/{*}/Krasnodar/{*}/07	Y_{10} ▼ $\mathbf{H_{10}}$; N_{397} ▼ $\mathbf{S_{397}}$; $\mathbf{T_{403}}$ ↓ A_{403}	Q_{39} ↓ $\boxed{\mathbf{L_{39}}}$; G_{41} ↓ $\boxed{\mathbf{R_{41}}}$; $\mathbf{L_{143}}$ ↓ V_{143}; P_{320} ▼ $\mathbf{L_{320}}$; K_{412} ↓ $\boxed{\mathbf{E_{412}}}$	no	no	I_{64} ↓ $\boxed{\mathbf{M_{64}}}$; D_{202} ▼ $\mathbf{G_{202}}$	M_{50} ▼ $\mathbf{V_{50}}$; I_{60} ▼ $\mathbf{T_{60}}$
A/duck/Novosibirsk/56/05 ↓ A/{*}/Rostov-on-Don/{*}/07	Y_{10} ▼ $\mathbf{H_{10}}$; K_{90} ▼ $\mathbf{R_{90}}$; A_{323} ↓ $\boxed{\mathbf{T_{323}}}$; A_{373} ▼ $\mathbf{T_{373}}$; N_{397} ▼ $\mathbf{S_{397}}$; $\mathbf{T_{403}}$ ↓ A_{403}	A_{46} ↓ $\boxed{\mathbf{V_{46}}}$; V_{63} ↓ $\boxed{\mathbf{L_{63}}}$; I_{102} ↓ $\boxed{\mathbf{V_{102}}}$; $\mathbf{L_{143}}$ ↓ V_{143}; Q_{288} ↓ $\boxed{\mathbf{R_{288}}}$	no	V_{68} ↓ $\boxed{\mathbf{I_{68}}}$; Q_{81} ↓ $\boxed{\mathbf{R_{81}}}$	D_{202} ▼ $\mathbf{G_{202}}$	M_{50} ▼ $\mathbf{V_{50}}$; I_{60} ▼ $\mathbf{T_{60}}$

B

Strains*	Amino acid substitutions** PB2	PB1	PB1-F2	PA	HA
A/{*}/05 ↓ A/{*}/{06–07}	no	no	no	no	N_{171} ↓ $\mathbf{D_{171}}$
A/duck/Novosibirsk/56/05 ↓ A/grebe/Novosibirsk/29/05	$\boxed{\mathbf{A_{69}}}$ ↓ E_{69}; L_{193} ↓ $\boxed{\mathbf{F_{193}}}$; A_{221} ↓ $\boxed{\mathbf{T_{221}}}$; K_{718} ↓ $\boxed{\mathbf{R_{718}}}$	V_{200} ↓ $\boxed{\mathbf{I_{200}}}$; Q_{294} ↓ $\boxed{\mathbf{H_{294}}}$	no	T_{618} ↓ $\boxed{\mathbf{A_{618}}}$	H_{371} ↓ $\boxed{\mathbf{R_{371}}}$
A/duck/Novosibirsk/56/05 ↓ A/Cygnus olor/Astrakhan/Ast05-2-{1-10}/05	$\mathbf{A_{69}}$ ↓ E_{69}	no	no	no	L_{8} ↓ $\boxed{\mathbf{F_{8}}}$; N_{170} ↓ $\boxed{\mathbf{D_{170}}}$
A/duck/Novosibirsk/56/05 ↓ A/grebe/Tyva/Tyv06-{1,2,8}/06	$\mathbf{A_{69}}$ ↓ E_{69}; M_{473} ▼ $\boxed{\mathbf{T_{473}}}$	T_{182} ▼ $\boxed{\mathbf{I_{182}}}$; V_{451} ▼ $\boxed{\mathbf{L_{451}}}$	no	no	V_{11} ▼ $\boxed{\mathbf{I_{11}}}$; A_{512} ▼ $\boxed{\mathbf{S_{512}}}$; N_{171} ▼ $\boxed{\mathbf{D_{171}}}$
A/duck/Novosibirsk/56/05 ↓ A/chicken/Moscow/2/07	M_{50} ↓ $\boxed{\mathbf{I_{50}}}$; I_{64} ▼ $\boxed{\mathbf{M_{64}}}$; $\mathbf{A_{69}}$ ↓ E_{69}; K_{126} ▼ $\boxed{\mathbf{R_{126}}}$; V_{338} ▼ $\boxed{\mathbf{I_{338}}}$	Y_{38} ↓ $\boxed{\mathbf{H_{38}}}$; I_{113} ▼ $\boxed{\mathbf{T_{113}}}$; L_{212} ↓ $\boxed{\mathbf{M_{212}}}$; E_{618} ▼ $\boxed{\mathbf{K_{618}}}$; M_{646} ↓ $\boxed{\mathbf{I_{646}}}$; S_{654} ↓ $\boxed{\mathbf{N_{654}}}$; S_{678} ↓ $\boxed{\mathbf{G_{678}}}$; A_{741} ↓ $\boxed{\mathbf{T_{741}}}$	no	V_{554} ▼ $\boxed{\mathbf{I_{554}}}$	T_{52} ▼ $\boxed{\mathbf{A_{52}}}$; N_{171} ▼ $\mathbf{D_{171}}$; L_{545} ↓ $\boxed{\mathbf{M_{545}}}$
A/duck/Novosibirsk/56/05 ↓ A/{*}/Krasnodar/{*}/07	$\mathbf{A_{69}}$ ↓ E_{69}; I_{354} ↓ $\boxed{\mathbf{V_{354}}}$; M_{473} ▼ $\mathbf{T_{473}}$; V_{495} ↓ $\boxed{\mathbf{I_{495}}}$	T_{182} ▼ $\mathbf{I_{182}}$; V_{451} ▼ $\mathbf{L_{451}}$	no	R_{213} ↓ $\boxed{\mathbf{K_{213}}}$; D_{272} ↓ $\boxed{\mathbf{E_{272}}}$	V_{11} ▼ $\mathbf{I_{11}}$; S_{12} ↓ $\boxed{\mathbf{N_{12}}}$; A_{102} ↓ $\boxed{\mathbf{S_{102}}}$; N_{171} ▼ $\mathbf{D_{171}}$; R_{178} ↓ $\boxed{\mathbf{I_{178}}}$; A_{512} ▼ $\mathbf{S_{512}}$
A/duck/Novosibirsk/56/05 ↓ A/{*}/Rostov-on-Don/{*}/07	I_{64} ▼ $\mathbf{M_{64}}$; $\mathbf{A_{69}}$ ↓ E_{69}; Q_{73} ↓ $\boxed{\mathbf{R_{73}}}$; K_{126} ▼ $\mathbf{R_{126}}$; V_{338} ▼ $\mathbf{I_{338}}$	I_{113} ▼ $\mathbf{T_{113}}$; E_{618} ▼ $\mathbf{K_{618}}$	no	R_{269} ↓ $\boxed{\mathbf{K_{269}}}$; S_{451} ↓ $\boxed{\mathbf{A_{451}}}$; V_{554} ▼ $\mathbf{I_{554}}$	Q_{31} ↓ $\boxed{\mathbf{H_{31}}}$; T_{52} ▼ $\mathbf{A_{52}}$; K_{56} ↓ $\boxed{\mathbf{R_{56}}}$; I_{99} ↓ $\boxed{\mathbf{N_{99}}}$; N_{171} ▼ $\mathbf{D_{171}}$

* Group of strains is shown using braces: designations common for all strains in the given group are shown outside the braces; variable part of designations is cited inside the braces; the asterisk "*" means "any designation". Only mutations that are found in all the strains of the given group are listed in the table.

** **Bold font** indicates substitutions in respect to HPAI / H5N1 / 2.2 consensus; $\boxed{\text{the frame}}$ – substitutions unique for Northern Eurasian strains (Tables 1–2) i.e. did not occur among Northern Eurasian strains previously; the frame with grey background – substitutions unique for all HPAI / H5N1 / 2.2 genotype (strains isolated in both Northern Eurasia and other places); «↓» – substitution that takes place in the strains of the given epizootic outbreak only; «▼» – substitution that takes place in the strains of both the given and late/previous epizootic outbreaks.

(*Cygnus olor*) (23). Swans had neurological disorders including inability to keep the neck or head raised (Fig. 5B), paralysis of the extremities (mainly legs), and depression. Because there are no human settlements in this part of the Volga River, no infections were detected among poultry. Migrating tufted ducks (*Aythya fuligula*), among which clinical features were not detected, are suspected to be the source of infection because the start of the epizooty coincided with tufted duck appearance. The sequences of the swan viruses, including A/Cygnus olor/Astrakhan/Ast05-2/2005, were closely related to the western Siberian strains (23) (Fig. 2) indicating distribution of virus through the Eastern European flyway of birds connecting western Siberia, the Russian Plain, Eastern Europe, the Middle East, and Africa (20).

Our fourth prediction was the return of virus in migrating birds from their overwintering places to northern Eurasia in the spring of 2006 with widening of the epizootic. Dramatic events occurred June 10–28, 2006, at the Uvs-Nur Lake, which is situated on the boundary of the Great Lakes Depression of Mongolia and Tyva

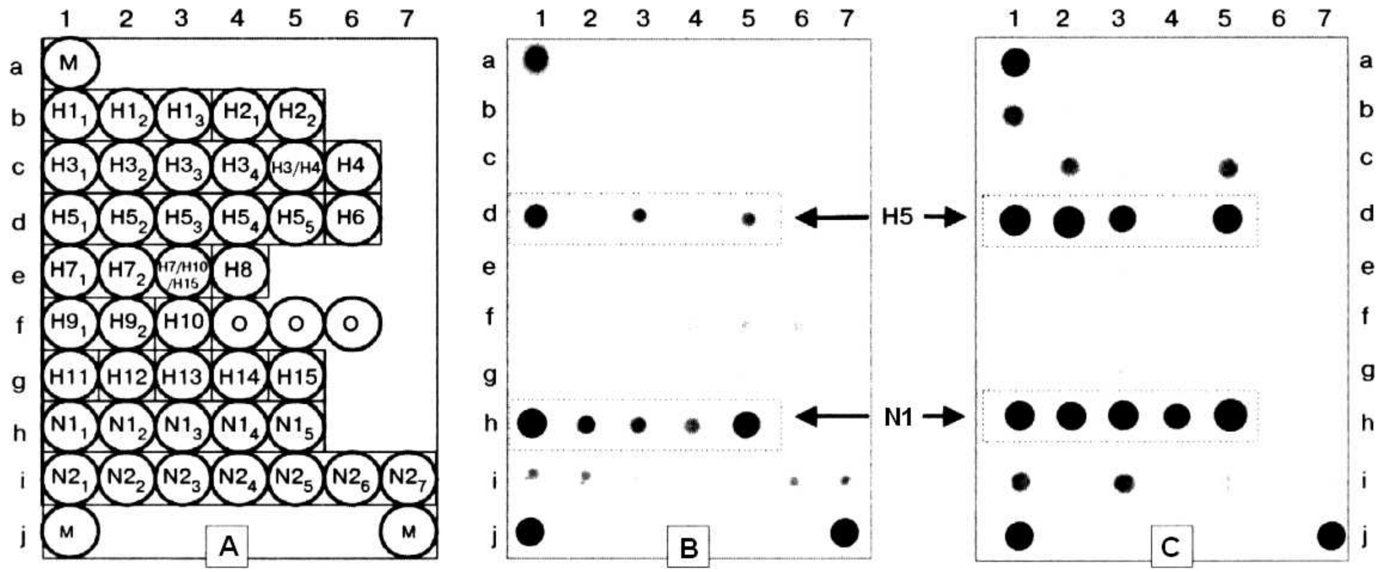

Fig. 4. Subtyping of HPAI H5/2.2 and 2.3.2 virus strains using biological microchips. (A) Biochip structure. (B) Hybridization pattern for A/chicken/Novosibirsk/64/2005 belonging to HA H5/2.2 (Qinghai-Siberian) genotype. (C) Hybridization pattern for A/chicken/Primorje/1/2008 belonging to HA H5/2.3.2 genotype.

Republic of Russia (24), where more than an estimated 3000 birds died in the Russian part of this lake, which is only about 1% of total area of the lake. The most affected species was crested grebes (*Podiceps cristatus*; Fig. 5C) as well as coots (*Fulica atra*; Fig. 5D) and cormorants (*Phalacrocorax carbo*). Terns and gulls were involved in the epizootic to significantly lesser extent. Absence of poultry farms in the vicinity of the Uvs-Nur Lake excluded outbreaks among poultry. The Tyva strains appeared to be the beginning of a new genetic lineage in the Qinghai-Siberian genotype that was designated as the "Tyva-Siberian" subgroup (Fig. 2), which was isolated not only in Siberia, but also in the Europe. It is believed that the Tyva-Siberian subgroup emerged in 2006 on nesting grounds of wild ducks in western Siberia. These birds were thought to have been infected in 2005 at the Great Lake Depression and at overwintering grounds in India. After the virus was introduced into the nesting place of northern Eurasia it was amplified.

A set of nine outbreaks occurred in the outskirts of Moscow beginning in February 2007. The occurrence of the outbreak at this time of year seems to preclude the participation of wild waterfowl in virus introduction or spread, and terrestrial wild birds were negative according to RT-PCR. Virus was isolated (see Table 1) from dead and sick poultry, and all the isolates were identified as HPAI H5N1/2.2 with high level of sequence similarity to the Qinghai-Siberian subgroup. This implied a common source of infection for all the outbreaks, and subsequent epidemiologic investigation demonstrated a link to the live bird markets in Moscow, where the affected farmers had purchased poultry several days before. The complete genome analysis of the prototype A/chicken/Moscow/2/2007 (27) revealed that the highest similarity occurred for the strains isolated in the Caucasian region during 2005–06 winter: A/cat/Dagestan/87/2006, A/Cygnus cygnus/Iran/754/2006, A/chicken/Krasnodar/01/2006, A/chicken/Adygea/203/2006 (similarity for nucleotide sequences of PB2 was 99.5%; PB1, 99.3%; PA, 99.7%; HA, 99.1%–99.4%; NP, 99.4%; NA, 99.1%–99.6%; M1, 99.5%–99.9%; NS1, 99.9%). The closest neighbor for A/chicken/Moscow/2/2007 was found to be A/Cygnus cygnus/Iran/754/2006. Later, this genetic subgroup of HPAI H5N1/2.2 was designated as "Iran–North Caucasian" (Fig. 2). Nevertheless, A/chicken/Moscow/2/2007 significantly differed from other Qinghai-Siberian strains. In 4 genes—PB2, PB1, HA, and NP—there were 12 unique amino acid substitutions (Table 3). Moreover, all eight amino acid substitutions in PB1 were unique at that time. This was the evidence of active circulation of virus before 2007. However, the specific origin of A/chicken/Moscow/2/2007 has not been officially identified, and it is suspected that virus was circulating in a small intermountain valley ecosystem in the North or South Caucasus in the winter of 2007 and that virus was introduced in the live bird market through contaminated poultry cages or grain.

In September 2007, an outbreak was detected on a chicken farm "Lebyazhje-Chepiginskaya" in the Krasnodar region (northeastern part of the Azov Sea basin). The virus isolates, A/chicken/Krasnodar/300/2007 from poultry and A/Cygnus cygnus/Krasnodar/329/2007 from sick whooper swan (*Cygnus cygnus*), found in the liman (shallow gulf) near the farm were closely related to each other (two synonymous nucleotide substitutions in PB1; two synonymous substitutions in PB2; one nonsynonymous substitution in M1; two nonsynonymous substitutions in NA; one nonsynonymous substitution in NS1) and belonged to the Iran–North Caucasian subgroup of the Qinghai-Siberian genotype (Fig. 2). Isolated strains contained 10 unique amino acid substitutions with respect to the Qinghai-Siberian consensus in PB2, PA, HA, NA, and NS1, which suggested that regional variants were continuing to emerge.

In December, 2007 infection of a poultry farm, Gulyai-Borisovskaya, in the Rostov region took place. Unfortunately, the infection was not reported in time and infected poultry manure was spread on adjacent fields, where wild terrestrial birds could be infected (28). This exposure is thought to have contributed to the infection of a number of species including (Fig. 5E) rooks (*Corvus frugilegus*), jackdaws (*Corvus monedula*), rock doves (*Columba livia*), common starlings (*Sturnus vulgaris*), tree (*Passer montanus*) and house (*Passer domesticus*) sparrows, and others. Surveillance of these species detected H5 virus by RT-PCR in 60% of pigeons and crows, in around 20% of starlings, and in 10% of tree sparrows without clinical features. These results were confirmed by virus isolation from wild birds and poultry (Table 1). Birds whose infection was confirmed by RT-PCR and virus isolation seemed reluctant to move and had ruffled feathers. On necropsy the birds were observed to have conjunctivitis and hemorrhages on lower extremities;

Fig. 5. Sick birds as the result of HPAI H5/2.2 virus infection. (A) Sick domestic duck (*Anas platyrhynchos domesticus*; south of western Siberia; July 2005). (B) Sick mute swan (*Cygnus olor*; mouth of Volga River; November 2005). (C) Sick great crested grebe (*Podiceps cristatus*; Uvs-Nur Lake; June 2006). (D) Sick coot (*Fulica atra*; Uvs-Nur Lake; June 2006). (E) Rooks (*Corvus frugilegus*) on the mixed fodder ground at the poultry farm Gulyai-Borisovskaya (Rostov region; December 2007). (F) Intestinal vessel plethora and changes in pancreas structure of infected rook (*Corvus frugilegus*) from the poultry farm Gulyai-Borisovskaya (Rostov region; December 2007).

hemorrhage in muscle, adipose tissue, intestine, mesentery, and brain; and changes in pancreas and liver structure (Fig. 5F). Wide involvement of wild terrestrial birds in virus circulation, presumably from the exposure to infected chicken manure, distinguished this outbreak from others (in particular virulence data of this outbreak are excluded from virulence trend analysis presented on Fig. 3). Genome analysis revealed the isolated strains belonged to the Iran–North Caucasian subgroup of the Qinghai-Siberian genotype (Fig. 2). They were phylogenetic similar to A/chicken/Moscow/2/2007 and had 13 unique amino acid substitutions with respect to Qinghai-Siberian consensus in PB2, PA, HA, NP, NA, and M2 (see Table 3).

Genetic stratification of the Qinghai-Siberian (2.2) genotype of HPAI H5N1 virus in northern Eurasia appeared to occur on the following ecologic model. In summer 2005, western Siberian cluster variants selected during epizootic outbreak at Kukunor Lake (Qinghai Province, China) were amplified in the summer nesting places. In winter 2005–06, HPAI H5N1/2.2 was under selection in two main overwintering areas: Africa, Transcaucasia, and Middle East (penetrating along Eastern and Western European flyways) as

Table 4. Possible influence of amino acid substitutions on their virulence (see Table 1, Fig. 3).[A]

Protein	Position	Virulence		Functional region of the protein	Influenced process of virus life cycle
		Increased	Decreased		
PB2	69	E[B]	A[B]	Domain of noncovalent binding with C-terminus of PB1	Formation and functioning of polymerase complex PB2-PB1-PA
	73	Q	R		
	221	A	T		
	473	M	T	NLS	Import of RNP into the nucleus
PB1	212	L	M	NLS	Import of RNP into the nucleus
	294	Q	H	Enzyme polymerase center	Functioning of polymerase complex PB2-PB1-PA
	451	V	L		
	618	E	K	Domain of noncovalent binding with N-terminus of PB2	Formation and functioning of polymerase complex PB2-PB1-PA
	678	S	G		
	741	A	N		
PA	213	R	K	NLS	Import of RNP into the nucleus
HA	512	A	S	Elongation fragment during fusion peptide disengagement	Virion and endosomal membrane fusion
	545	L	M	C-terminus transmembrane domain	
NP	10	Y	H	NLS	Import of RNP into the nucleus
	323	A	T		
	373	A	T		
	397	N	S		
	403	T	A		
NA	29	M	I	N-terminus transmembrane domain	New virion assembling
M2	68	I	V	C-terminus cytoplasm domain	Interaction between M2 and M1
	81	R	Q		
NS1	64	I	M	Unknown	Interaction with signal systems of infected cell
	202	D	G		
NS2	50	M	V	Terminus of α-helix N2	Export of RNP from the nucleus
	60	I	T	Base of α-helix C1	

[A]NLS = nuclear location signal; RNP = ribonucleoproteide.
[B]Letters are abbreviations for amino acids.

well as India and Central Asia (penetrating along Indian-Asian flyway) (20). The first overwintering area could be the source for the Iran–North Caucasian subgroup whereas the second could be the source for the Tyva-Siberian subgroup. Returning back to nesting areas in northern Eurasia in the spring of 2006, wild birds started the amplification of the variant subgroups, which were naturally mixed in the south of the Russian Plain, on the crossroad of different flyways.

In April 2008, the second breach of HPAI H5N1 into northern Eurasia emerged in Primorje (in the Far East) and was linked with another genotype 2.3.2 (29). The epizootic originated from unvaccinated poultry in outermost backyard of Vozdvizhenka village, located by a small river and watery meadow, where poultry often interacted with wild waterfowl. One initial theory of introduction to poultry was from exposure to hunted ducks, but the direct interaction of wild birds with poultry seems more likely. The isolates (Table 1) from dead chickens and common teal (*Anas crecca*) collected in the vicinity of epizootic farms were identical and indicated a direct role of migrating birds in virus introduction. Common teal, which appeared to be the most likely source of infection for poultry, had no obvious behavior changes but on necropsy they did have hemorrhagic lesions on the intestines. It is interesting to underline that it was common teals that were the source of isolation of H5 strains in Primorje in autumn 2001. Common teals migrate for a long distances, so the hypothesis is that HPAI H5N1/2.3.2 variants migrated from the Far East, possibly southern China, Vietnam, or Laos. The HPAI H5N1 virus was widely distributed in Primorje in spring of 2008; according to RT-PCR, 26% of wild ducks in the Suifun River–Khanka Lake lowland were infected (29). However, during monitoring in autumn of 2008 we were not able to find HPAI H5N1. Nevertheless, emergence of HPAI H5N1 virus in Primorje creates a new type of HPAI stratification in northern Eurasia (genotype 2.2 in the eastern areas and 2.3.2 in the western sectors of this subcontinent) as well as a threat of HPAI introduction into North America.

Comparison of data from Table 3 and Fig. 3 allows localization amino acid substitutions that are possibly associated with the level of *in vitro* replication potential. Possible functional roles of such amino acid substitutions are suggested in Table 4.

Thus, using the example of HPAI H5N1 evolution in ecosystems of northern Eurasia (2005–08) we have attempted to illustrate the concept that emerging and reemerging infections such as HPAI can not be completely eliminated in the near future. Nevertheless, ecologic monitoring and adequate data analysis could lead to well-timed predictions, which can provide opportunities to mitigate disease outbreak consequences.

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